

Isolation, genomic organization, and expression analysis of *Men1*, the murine homolog of the MEN1 gene

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Abstract. The mouse homolog of the human MEN1 gene, which is defective in a dominant familial cancer syndrome, multiple endocrine neoplasia type 1 (MEN1), has been identified and characterized. The mouse *Men1* transcript contains an open reading frame encoding a protein of 611 amino acids which has 97% identity and 98% similarity to human menin. Sequence of the entire *Men1* gene (9.3 kb) was assembled, revealing 10 exons, with exon 1 being non-coding; a polymorphic tetranucleotide repeat was located in the 5'-flanking region. The exon-intron organization and the size of the coding exons 2–9 were well conserved between the human and mouse genes. Fluorescence in situ hybridization localized the *Men1* gene to mouse Chromosome (Chr) 19, a region known to be syntenic to human Chr 11q13, the locus for the MEN1 gene. Northern analysis indicated two messages—2.7 kb and 3.1 kb—expressed in all stages of the embryo analyzed and in all eight adult tissues tested. The larger transcript differs from the smaller by the inclusion of an unspliced intron 1. Whole-mount in situ hybridization of 10.5-day and 11.5-day embryos showed ubiquitous expression of *Men1* RNA. Western analysis with antibodies raised against a conserved C-terminal peptide identified an approximately 67-kDa protein in the lysates of adult mouse brain, kidney, liver, pancreas, and spleen tissues, consistent with the size of human menin. The levels of mouse menin do not appear to fluctuate during the cell cycle.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by tumors of the parathyroids, enteropancreatic endocrine tissues, and the anterior pituitary (Metz et al. 1994). The MEN1 locus was mapped to human Chr 11q13 by linkage analysis (Larsson et al. 1988), and the responsible MEN1 gene was identified by positional cloning (Chandrasekharappa et al. 1997). The human MEN1 gene is organized into 10 exons (the first being untranslated) and is ubiquitously expressed as a 2.8-kb transcript. This transcript encodes a 610-amino acid product, termed menin, which exhibits no apparent similarities to any known proteins. Over 200 independent germline and somatic mutations spread throughout the coding region of

menin have been described (Marx et al. 1999). Somatic mutations are observed to a variable extent in certain sporadic tumors: parathyroid adenoma, gastrinoma, insulinoma, and lung carcinoma (Marx et al. 1999). MEN1 appears to be a typical tumor suppressor gene: lack of menin owing to inactivation of both alleles probably leads to the development of tumors.

Although no murine syndrome similar to MEN1 has been reported to date, here we detail the identification of the mouse MEN1 homologous gene, *Men1*, on mouse Chr 19. The cDNA sequence, genomic organization, RNA and protein expression patterns are presented and discussed.

Materials and methods

Screening BAC library. The entire *Men1* cDNA sequence was assembled by sequencing the insert in a cDNA clone (IMAGE clone ID 557658). DNA pools from a BAC library prepared from 129Sv/cJ7 mouse DNA (Research Genetics, Huntsville, AL) were screened by PCR with primers (5'-GCTGAAGGCGCCAGAAAGACG-3' and 5'-CTGAGCGGTGAATCGGGCATAGAG-3') designed from the mouse *Men1* cDNA sequence. Three BAC clones, 331J21, 331K21, and 7D23, were isolated. The size of the inserts was determined by pulsed-field gel electrophoresis of *NotI*-digested BAC DNAs as described earlier (Guru et al. 1997b).

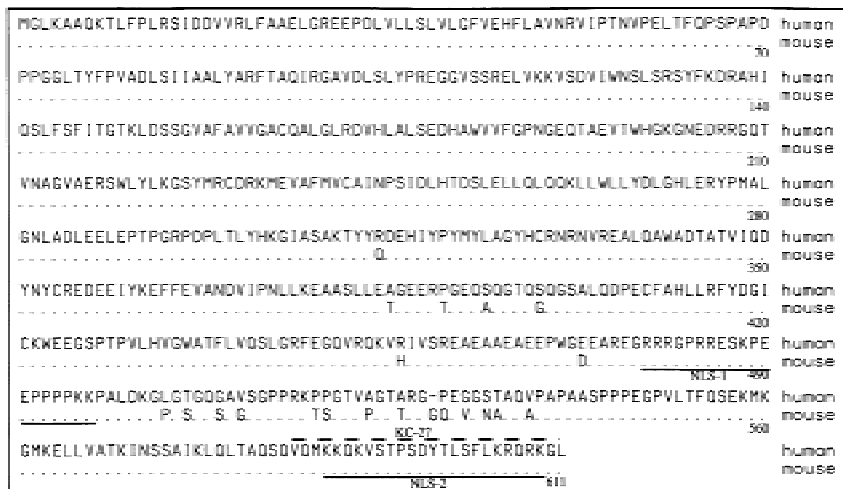
Subcloning and sequence analysis. DNA isolated from the BAC clone 7D23 was subjected to partial digestion with *Sau3A1* to generate fragments of approx. 10–12 kb. The fragments were cloned into the *Bam*HI site of the plasmid pBluescriptII KS+ (pBSIIKS+). Colonies were analyzed by PCR with STSs designed for both the 5'- and 3'-ends of the *Men1* cDNA in order to identify the clones containing the entire gene. One clone with an approximate 10-kb insert containing the entire *Men1* gene was chosen for subsequent sequence analysis and genomic characterization.

Primers, UP1 (GACATCCATGGCTACACAGAAAAACCC) and LP1 (GCCTGTGTAAAGGGAAGAAGACAGAGAGAGT), generating a 260-bp product, were used for the PCR amplification of the (AAAG)₁₀ repeat alleles from mouse genomic DNA.

Northern analysis. A 900-bp insert representing the 3'-end of the *Men1* cDNA clone (IMAGE clone ID. 402210) was released by digestion of the plasmid DNA with *EcoRI* and *NotI*, labeled with a random primer labeling kit (Amersham) and hybridized to a mouse multiple tissue Northern blot (#7762-1) and embryo blot (#7763-1) (Clontech, Palo Alto, CA) as described earlier (Guru et al. 1997a). For the 5'-end probe, a 299-bp PCR product representing most of intron 1 was amplified from the BAC clone 331J21.

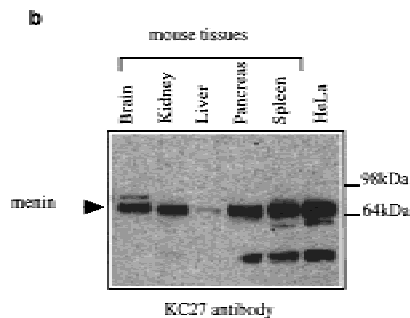
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Fig. 1. a) Comparison of human and mouse menin sequences. Alignment of the amino acid sequence was carried out using Multiple Sequence Alignment program of DNASTAR. The human and mouse menin sequences are shown on the top and bottom respectively. An identical amino acid in mouse menin is indicated by a dot. The numbering of amino acid refers to the mouse protein sequence. The C-terminal 27 aa sequence used for producing a rabbit antibody (KC-27) is indicated with a broken line on top of the sequence. The two nuclear localization signals (NLS-1 and NLS-2) mapped in human menin are underlined. **b)** Western analysis of protein lysates from five adult mouse tissues and HeLa cells (human) probed with KC27 antibodies. The location of menin is indicated on the left, and those of size markers on the right.



Western analysis. Mouse tissues were homogenized in Tris-HCl buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 0.5% NP-40, 100 μ g/ml AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], 1 μ g/ml aprotinin/leupeptin) and centrifuged at 20,000 g for 1 h. The supernatant was mixed with Laemmli buffer, boiled for 5 min, and then the proteins (40 μ g for each tissue and 10 μ g for HeLa cell lysate) were separated on a 10–20% SDS gel. Production of KC27 antibodies in rabbits to a C-terminal human menin 27 aa peptide and routine Western analysis procedures were as described previously (Guru et al. 1998).

Fluorescence in situ hybridization (FISH). Preparation of metaphase chromosomes from lymphocytes obtained from mouse spleen, hybridization conditions, and analysis of fluorescent signals were as described previously (Pecker et al. 1996). The BAC clone (331J21) containing the mouse *Men1* gene was labeled by nick translation with biotin-conjugated dUTP. In order to facilitate chromosome identification, a digoxigenin-labeled mouse Chr 19-specific painting probe was used for co-hybridization. The biotinylated probes were detected by incubation with avidin-FITC, and the digoxigenin sequences were detected with mouse anti-digoxin and goat anti-mouse conjugated to TRITC (Sigma Chemical Co., St. Louis, Mo.). Chromosomes were counterstained with DAPI.

Whole-mount mouse embryo in situ hybridization. Non-radioactive in situ hybridization was performed on whole embryos [10.5 and 11.5 days post coitum (p.c.)] and cryosections (12.5 days p.c.) prepared as previously described (Kos et al. 1999). Embryos were obtained from matings of FVB mice. Noon of the plug day was considered 0.5 days p.c., but embryonic ages were confirmed by comparison of somite number and external features with descriptions by Kaufman (1992). Sense and antisense digoxigenin-labeled riboprobes were generated from linearized templates by in vitro transcription with T3 or T7 RNA polymerase. A plasmid (IMAGE clone ID. 402210) with an insert representing the 900-bp 3'-end of the *Men1* cDNA was used as a template. For antisense probe, the plasmid was

linearized with *EcoRI* and transcribed with T3 RNA polymerase. For sense probe (negative control), the plasmid was linearized with *NotI* and transcribed with T7 RNA polymerase.

Cell synchronization. Synchronization of cells, preparation of protein lysates, and Western analysis were as described earlier (Brown et al. 1997). Briefly, NIH-3T3 cells were synchronized in G_0 by culturing the cells for 96 h under reduced serum [0.5% fetal calf serum (FCS)] conditions in Dulbecco's Modified Eagle Medium (DMEM). To collect cells synchronized in G_1 , serum-starved cells were replated in complete growth medium (DMEM supplemented with 10% FCS) for 6 h. Mitotic (M) cells were obtained by culturing in complete growth medium supplemented with colcemid (0.1 μ g/ml) for 24 h, and mitotic cells were collected by mitotic shake-off. Extracts of NIH-3T3 cells in log phase growth (Log) were obtained from asynchronous cultures grown in complete growth medium. Aliquots (1×10^6 cells) of asynchronous and synchronized cell populations were stained with propidium iodide and analyzed by flow cytometry to confirm their stage in cell cycle.

Results

Mouse *Men1* cDNA sequence and its encoded 611 aa mouse menin protein. A search of the NCBI EST database (dbEST) with human *MEN1* cDNA sequence revealed the availability of several mouse cDNA clones with similar sequences. Analysis of the homologous regions in the available EST sequences and the sizes of the clone inserts resulted in identification of the longest cDNA clone (IMAGE clone ID. 557658). A 2984-bp sequence (Genbank acc. no. AF109389) was assembled for the entire cDNA insert in this clone. The sequence includes an ORF encoding the putative mouse menin protein of 611 aa, one amino acid longer than that of human menin (610 aa). The encoded mouse protein shows 97% identity

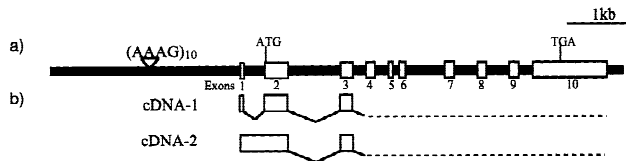


Fig. 2. a) Exon-intron organization of the *Men1* gene. The 9330-bp genomic sequence is shown as a thick horizontal line, and the locations of the 10 exons (open boxes), the tetranucleotide repeat (AAAG)₁₀ in the 5'-flanking region, the initiation codon ATG in exon 2, and the termination codon (TGA) in exon 10 are shown. The exon numbers are indicated below. **b)** A diagram depicting the differences near the 5'-end of the two types of transcripts: cDNA-1, completely spliced 2592-bp transcript. cDNA-2, a 2984-bp transcript with unspliced intron 1. The dotted line indicates that there are no changes in the remaining part of the two transcripts.

with and 98% similarity to human menin sequence (Fig. 1a). Antibodies (KC27) raised against a conserved C-terminal peptide (indicated in Fig. 1a) were used for the identification of mouse menin by Western analysis of lysates from adult mouse brain, kidney, liver, pancreas, and spleen tissues. All the tissues showed a protein of approx. 67 kDa, similar in size to that of human menin (Guru et al. 1998) from HeLa cell lysate (Fig. 1b).

***Men1* gene sequence and exon-intron organization.** Three BAC clones were isolated by screening a mouse BAC library with an STS specific for the *Men1* cDNA sequence. The inserts in clones 331J21 and 331K21 were similar in size (280 kb), whereas that of the clone 7D23 was 120 kb. In addition to their similar size, the identical *NotI* enzyme restriction pattern and their adjacency in the library suggested that the clones 331J21 and 331K21 are likely to be copies. PCR analysis with primers amplifying both ends of the mouse *Men1* cDNA sequence revealed that all BAC clones had the entire *Men1* gene.

In order to obtain genomic sequence of the *Men1* gene, a plasmid library was prepared by cloning *Sau3A*I digests of the BAC clone 7D23 into the *Bam*H1 site of pBSIISK+. PCR analysis of plasmid clones identified a clone with an approximate 10-kb insert containing the entire *Men1* gene, and a 9286-bp sequence (Genbank acc. no. AF109390) of the mouse gene was assembled by sequencing this insert. The genomic sequence revealed that, similar to the human gene, the mouse gene also consists of 10 exons, and the locations of the initiation (ATG) and the termination codons are in exons 2 and 10, respectively (Fig. 2a). The sizes of the intervening exons 2–9 are identical in human and mouse. Comparison of homologous mouse ESTs in the dbEST database to the assembled *Men1* genomic and full-length cDNA sequences indicated two types of cDNA clones. One resembled the human, and transcripts with an unspliced intron 1 (362 bp) along with the otherwise fully processed transcripts contributed the second type of mouse *Men1* cDNA clones (Fig. 2b). However, the 611-aa ORF with the translation initiation point (ATG) in exon 2 remained unaltered in both types of messages. A single variant polyadenylation signal (GATAAA) is located 20 nucleotides upstream of the poly-A stretch in all seven *Men1* cDNA clones analyzed.

A tetranucleotide repeat (AAAG)₁₀ was observed about 1.5 kb upstream of the 1st exon. PCR amplification of the repeat region in DNA samples from various strains of mice revealed four different alleles indicating that this repeat is polymorphic. Allele sizes in *DBA/2J*, *FVB/NJ*, and *M. spretus* are different from each other. The (AAAG)₁₀ allele was observed in C57bl/6J, A/J, and 129/SvJ mice (data not shown).

Chromosomal localization of the Men1 gene by FISH. Chromosomal localization of the *Men1* gene was carried out by FISH analysis of mouse metaphase chromosomes. A biotin-labeled

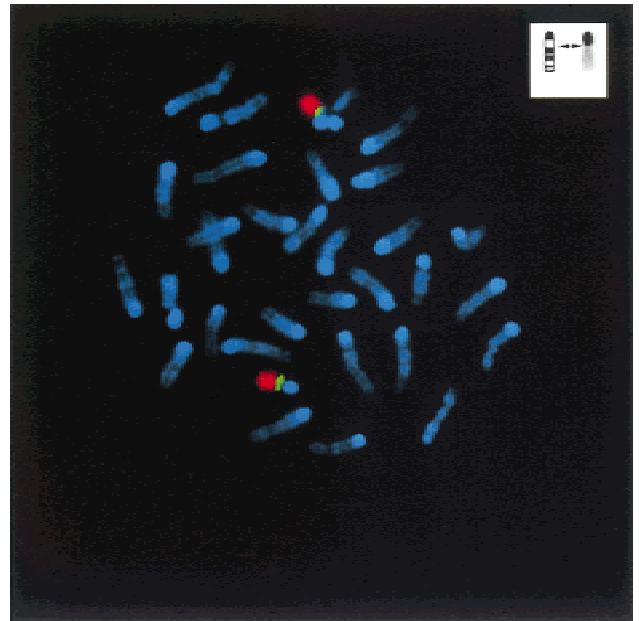


Fig. 3. Fluorescent in situ hybridization of *Men1* gene to mouse metaphase chromosomes. Labeled BAC clone 331J21 DNA (green signal) and Chr 19-specific painting probes (red signal) were cohybridized to mouse metaphase chromosomes. The location of the green signal owing to hybridization of *Men1* containing BAC DNA on the proximal region of Chr 19, between cytogenetic bands B and C2, is depicted in the inset.

331J21 BAC DNA probe was cohybridized with a digoxigenin-labeled mouse Chr 19-specific painting probe. The BAC clone (green signal) hybridized to the proximal region of mouse Chr 19 (red signal; Fig. 3). This region Chr 19 is syntenic with human pericentric Chr 11q13 containing the *MEN1* gene. The BAC clone hybridization signals were observed exclusively at Chr 19.

***Men1* gene expression by Northern analysis.** A multiple-tissue Northern blot representing RNA from eight adult mouse tissues (Clontech) was probed with a labeled 900-bp cDNA insert representing the 3'-end of the *Men1* cDNA. The presence of two messages, sized at 2.7 kb and 3.1 kb, was observed in all tissues, although the extent of expression between the tissues varied considerably (Fig. 4a). The expressions in heart, spleen, and skeletal muscle were reduced compared with brain, lung, liver, kidney, and testis. Both messages appear to be expressed in nearly equal amounts in all tissues except for brain, where the longer transcript was prominent. In order to resolve the nature of the two messages, a 299-bp probe generated by PCR amplification of most of intron 1 was hybridized to the same Northern blot. Only the longer 3.1-kb message hybridized to the intron 1 probe, indicating that the longer message originated owing to an unspliced intron 1 (Fig. 4b).

Analysis of *Men1* gene expression during embryonic development with a Northern blot with RNAs from 7-, 11-, 15-, and 17-day embryos showed that both messages (2.7 kb and 3.1 kb) were expressed at all these stages (Fig. 4c). Minimal variation was observed as to the relative amounts of the two messages.

Whole-mount in situ hybridization. In situ hybridization was used to examine the distribution of *Men1* mRNA during mouse embryogenesis. Wild-type embryos were examined by whole-mount in situ hybridization at 10.5 and 11.5 days p.c. and by hybridization to cryosections at 12.5 days p.c. At all ages examined, *Men1* mRNA appeared to be ubiquitously expressed throughout the embryos. Hybridization result from the 11.5 day p.c. embryo is shown in Fig. 5. Examination of cross sections indicated a higher level of

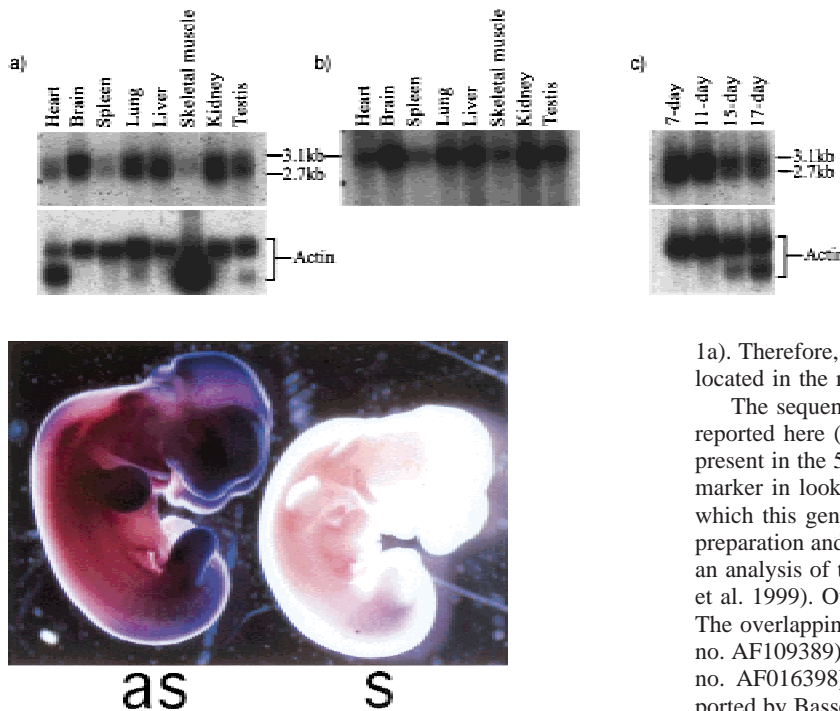


Fig. 5. Expression of *Men1* in mouse embryos by whole-mount in situ hybridization. Antisense (as) and sense (s; control) RNA probes generated by T7 and T3 RNA polymerase with a cDNA clone representing the 900 bp from the 3'-end of *Men1* cDNA were hybridized to an 11.5-day embryo.

expression in cranial ganglia, sensory ganglia, and neural tube than observed in adjacent tissues.

Mouse menin expression in the cell cycle. Cultures of the murine cell line NIH-3T3 were synchronized at various points in the cell cycle (G_0 , G_1 , and M phase) and, along with asynchronous cultures of cells in log-phase growth (Log), were analyzed by Western analysis with KC27 antibodies. The menin levels in all these lysates were equivalent, indicating that menin expression does not fluctuate during the cell cycle.

Discussion

The mouse homolog (*Men1*) of the human MEN1 gene, located as expected at the syntenic region on Chr 19, has been isolated and characterized. A similar exon-intron structure, identical sizes of coding exons, 88% similarity of the coding nucleotide sequence, and 97% identity of the encoded proteins indicate that the *Men1* gene is highly conserved through evolution. In addition to an extra amino acid in the mouse menin, there are only 20 locations where the amino acids differ in the entire length of the two proteins (Fig. 1a). It is interesting to note that none of the 46 amino acids involved in disease-associated germline and/or somatic missense mutations or inframe deletions are different in mouse menin, showing the importance of conservation in defining the structural and functional role of menin. The two nuclear localization signals (Guru et al. 1998) have been completely conserved in mouse (Fig.

Fig. 4. *Men1*, gene expression by Northern blot analysis. **a)** A multiple-tissue Northern blot (Clontech) with RNA from eight adult mouse tissues (indicated on the top) was hybridized to a 900-bp radiolabeled probe representing the 3'-end of the *Men1* cDNA. Size and location of the longer (3.1 kb) and the smaller (2.7 kb) messages are indicated. The signals obtained from a beta-actin probe used as a control for RNA loading on this blot are shown at the bottom. **b)** The same blot was probed with a radiolabeled 299-bp probe representing intron 1. Hybridization of this probe only to the longer 3.1 kb message is indicated. **c)** Hybridization of a Northern blot containing RNA from 7-, 11-, 15- and 17-day mouse embryos with the 900-bp *Men1* cDNA probe as in (a). The locations of the 3.1-kb and 2.7-kb messages are shown. Hybridization of this blot to a control beta-actin probe is shown below.

1a). Therefore, mouse menin is also expected to be predominantly located in the nucleus.

The sequence of the entire mouse *Men1* gene (9.3 kb) is also reported here (AF109390). A polymorphic tetranucleotide repeat present in the 5'-flank of the *Men1* gene may be a helpful genetic marker in looking for loss of heterozygosity in mouse tumors in which this gene might play a role. While this manuscript was in preparation and revision, two other groups independently reported an analysis of the mouse *Men1* gene (Stewart et al. 1998; Bassett et al. 1999). Our results are in agreement with these two reports. The overlapping sequences of *Men1* reported here (Genbank acc. no. AF109389) and those of Stewart and associates (Genbank acc. no. AF016398) are identical, but differ from the sequences reported by Bassett and coworkers (Genbank acc. no. AF072755) at three locations. These differences are: at codons 457 (I changing to M), 466 (E changing to G), and 512 (S changing to L). In these three positions, the sequences reported here are conserved and identical to that of the human menin sequence. The sequences we assembled from seven independent cDNA clones for the 3' end indicate a GATAAA sequence, located 19 nt upstream of the polyA stretch, as the likely polyadenylation signal.

The mouse *Men1* gene is expressed in at least two alternate forms—an additional isoform arises owing to alternative splicing of intron 1. Both types of messages appear to be expressed nearly equally. The ORF and, therefore, the sequence of the encoded mouse menin are unaltered in these two messages, but alternative translational efficiency is possible.

Northern analysis indicates widespread expression of *Men1* in all embryonic stages (7-day to 17-day) and in all eight adult tissues tested. The significance of the modest expression level differences between different tissues and at different embryonic stages, if any, is unclear.

The distribution of *Men1* mRNA was also examined by whole mount in situ hybridization. In mouse embryos, *Men1* was ubiquitously expressed, with somewhat higher levels in neural tissues. The ubiquitous expression and early embryonic expression of mouse menin is suggestive of its fundamental role in a widespread biological function. The molecular basis for the development of neoplasia in restricted endocrine tissues in patients with MEN1, despite ubiquitous expression of the gene, remains to be determined. It is possible that other genes are able to compensate for lost MEN1 function in the unaffected tissues. Analysis of organ function in mice with engineered alterations in *Men1* will be useful for understanding the role of *Men1* in development and disease.

Antibodies raised against a C-terminal peptide that is identical in human and mouse allowed identification of mouse menin by Western analysis (Fig. 1b). The size of the mouse protein, as expected, is similar to that of the human, and levels of menin do not appear to fluctuate during the cell cycle (Fig. 6). These anti-

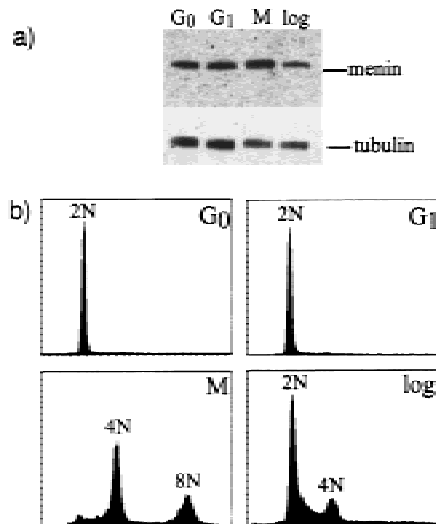


Fig. 6. Mouse menin levels in NIH-3T3 cells at different stages of cell cycle. **a)** Using the KC27 antibody, menin levels were analyzed by Western blot (whole cell extract; 10 μ g protein each lane) from extracts derived from NIH-3T3 cells synchronized in the G₀, G₁, and Mitotic (M) phases of the cell cycle, as well as cells from asynchronous cultures in log phase of the cell growth (Log). The same blot was probed with a tubulin antibody to confirm that equivalent amounts of protein were present in each lane. **b)** Aliquots of cells were stained with propidium iodide and subjected to flow cytometric analysis. Shown are histograms where cell number is plotted on the Y-axis and DNA content on the X-axis, confirming the cell cycle stage of the synchronized cell populations analyzed in **a**.

bodies may be useful in future biochemical characterization of mouse menin.

This characterization of the mouse *Men1* gene and its protein product should now set the stage for development of a mouse model of the human disease.

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